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Research Article

Efficacy of plant growth promoting rhizobacteria in *in vitro* inhibition of *Xanthomonas axonopodis* pv. *glycines* and prevention of bacterial pustules of soybean in the field

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ABSTRACT: Twelve different plant growth promoting rhizobacteria (PGPRs) belonging to *Pseudomonas fluorescens* and *Bacillus subtilis* were tested for their efficacy against bacterial pustules of soybean (*Glycine max*) caused by *Xanthomonas axonopodis* pv. *glycines* both in laboratory and field conditions. PGPR-10 (*P. fluorescens*), PGPR-3 (*B. subtilis*), PGPR-4 (*B. subtilis*) and PGPR-12 (*P. fluorescens*) showed maximum bacterial growth inhibition *in vitro* (more than 30mm inhibition zone) and increased seed germination (more than 92%). Highest seed germination (above 95%) occurred with the treatment of PGPR-4 (*B. subtilis*), PGPR-10 (*P. fluorescens*), PGPR -1 (*B. subtilis*), PGPR-7 (*P. fluorescens*), PGPR-11 (*P. fluorescens*) and PGPR-5 (*B. subtilis*), while plant vigor increased with PGPR-12 (*P. fluorescens*), PGPR-5 (*B. subtilis*), PGPR-11 (*P. fluorescens*) and PGPR-3 (*B. subtilis*). Under field conditions, PGPR-12 (*P. fluorescens*) and PGPR-5 (*B. subtilis*) resulted in decrease of per cent disease index and increase in plant yield compared to other PGPR strains.

KEY WORDS: Disease management, *Glycine max*, *Xanthomonas axonopodis* pv. *glycines,* PGPRs

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INTRODUCTION

The soybean [*Glycine max* (L.) Merr.] is an important oilseed crop of India and grown in 9.69 million ha with 9.05 million tonnes seed yield (FAO, 2008). The bacterial pustules caused by *Xanthomonas axonopodis* (syn. *campestris)* pv. *glycines,* a foliar disease with a widespread occurrence in India (Patel, 1972), cause premature defoliation, reduced seed size and number in beans. High temperature and humidity accompanied by sporadic heavy rain all favor disease development. The effect of bacterial pustules on the growth and development of soybean depends on infection levels. Severe incidence ranging between 10.5 and 77.8% has been reported to cause yield losses up to 37.7% (Singh and Jain, 1988) of which maximum loss up to 50-80% occurs due to cotyledon infection while 18-30% loss is due to leaf infection and reduction of seed number and size. The farmers have lately shown aversion to the cultivation of this crop because of the severe yield losses. Difficulties have been encountered in the management of bacterial diseases of plants because of the availability of few bactericides.. Further, antibiotics are expensive and not very efficacious against all the bacterial pathogens. Considerably great efforts have been made in the recent past to evolve non-conventional and environmentally safe approaches including biological, cultural and integrated pest management for plant disease management. Consequently, the present study was undertaken to screen 12 plant growth promoting rhizobacteria (PGPRs) *in vitro* and best selected treatments were further evaluated under field condition to manage the crop losses.

MATERIALS AND METHODS

Management of disease

Twelve PGPR isolates (Table 1) were tested for their efficacy against the disease both in laboratory and field conditions (Satish *et al.,* 1999).

In vitro *assay of PGPRs*

The putative PGPRs isolated from cluster bean rhizosphere and phyllosphere (Sain and Gour, 2007; 2009) (1-6, *Bacillus subtills* and 7-12 *Pseudomonas fluorescens*)

were tested by disc diffusion technique on bioassay medium (peptone 10 g, beef extracts 3 g, yeast extracts all bacteriological type (Hi-Media) 5 g, agar 20 g.) previously seeded with 48 h old bacterial pathogen (*Xanthomonas axonopodis* pv. *glycines*) cultures (1 ml/ 250 ml) in Petri plates (30 ml). The paper dishes discs (10 mm dia) of Whatman No.1 filter paper were dipped into the 48 h old PGPRs cultures suspension of 10^6 and 10^4 cfu ml^{-1} concentration, separately by using a standard stock of 10^8 cfu ml^{-1} (OD_{540}=0.2) and placed on medium in Petri plates (3 discs/plate) as for chemical tests. The growth inhibition (%) was recorded after 72 h of incubation following the formula. I = C-T/Cx100. Where, I = inhibition %, C = colony diameter in control plate (mm), T = colony diameter in treatment plate (mm) and inhibition data were analyzed by analysis of variance (ANOVA) and treatments were separated by F-protected (P=0.05) least significant difference (LSD) test.

Effect of seed treatment on seed germination and plant vigor index (PVI)

The seeds of soybean which were previously inoculated with pathogen (6 h seed soaking) were treated with PGPRs separately (1 h seed soaking) and plated on three layers of moistened sterilized blotter paper in Petri pates (10-20 seeds Petri $plates^{-1}$) at 28±2° C. Untreated seeds served as control. 10 days after incubation seed germination % was recorded and seedling vigor (PVI) was calculated by following formula. PVI = (mean epicotyl length + mean hypocotyl length) x % germination (Baki and Anderson, 1973). The experiment was repeated thrice. ANOVA was used to determine the effects of treatments. The angular transformation was used to stabilize variances. Complete randomized block design test was used for least significant difference to separate means after ANOVA at $P = 0.05$.

Efficacy of PGPRs under field conditions

Field experiments were carried out at Rajasthan College of Agriculture farm, Udaipur during two consecutive crop seasons (2000 and 2001) in randomized block design with three replications. Three antibiotics, five plant extracts and five antibacterial biocontrol agents which showed better performance in the *in vitro,* with respect to seed germination and PVI tests at different concentrations were used to treat seeds (pathogen-inoculated), separately and sown in 2 x 2 meter plots in four replications, separately. Two weeks after germination crop was spray inoculated thrice at an interval of 12 h with bacterial inoculum (10^6 cfu/ml) under maintained moist condition (by frequently water spraying during daytime) for 3-4 days. The three sprays at the interval of 10 days of chemicals (300 ppm), plant extracts (50%) and antibacterial PGPRs (10^8 cfu ml^{-1}) were given three days after last inoculation coinciding with the time of first appearance of the symptoms. The disease intensity/index was recorded after 15-20 days of last spray. Per cent disease control (PDC) was calculated by the following formula: PDC = (infection index in control plots – infection index in treatment plots/ infection index in control plots) x 100. Per cent increase in yield was recorded by the following formula: Per cent increase in yield = (yield in treatment –yield in control/ yield in control) x 100. The average data of three replications of both the years 2000 and 2001 were pooled, and analysis of variance (ANOVA) was used to determine the effects of treatment on the percentage of disease incidence, PDC and total yield. The angular transformation was used to stabilize variances. Randomized block design least significant difference test was used to separate means after ANOVA at $P = 0.05$.

RESULTS AND DISCUSSION

In vitro assays of PGPRs

Among the 10 putative PGPR isolated from cluster bean rhizosphere and phyllosphere (1–6, *Bacillus sp.* and 7–12 *Pseudomonas sp.*) PGPR–10 showed significantly (LSD < P 0.05) maximum inhibition diameter (33.5 mm) at 10^8 cfu ml^{-1} concentration (Table 1), followed by PGPR–3, PGPR–4, and PGPR–12. However, PGPR–8, PGPR–7, PGPR–5, and PGPR–11 were also inhibitory to the bacterial growth to a certain extent (22–28 mm).

Effect of seed treatment on seed germination and plant vigor index (PVI)

Of the PGPRs evaluated, almost all PGPRs improved the overall growth and % germination and at the same time reduced the pathogenic effects of the bacterial strain. A significant (LSD < P 0.05) increase in % germination (> 98%) was recorded in seeds treated with PGPR–12, PGPR–4, PGPR–1, PGPR–7 and PGPR–11 compared to both the controls. Average $plant^{-1}$ dry weight was significantly higher in treatments with PGPR–3, PGPR–12, PGPR–4, and PGPR–11, respectively but there is no significant difference amongst these treatments (Table 2). However, the PVI was significantly higher by the treatment of PGPR–12 followed by PGPR–5, PGPR–11 and PGPR–3 compared to other treatments and controls.

These results obviously indicated that PGPRs possess the plant growth promoting activity, which not only acts as antibacterial, but also enhance PVI and seed germination. *B. subtilis*, *Pseudomonas* spp and *Bacillus* sp. have been reported to be highly efficient against *X. campestris* pv. *malvacearum* (Assis *et al.*, 1995; Mondal *et al.*, 1999), *B. subtilis* BO34 isolated from rice

Table 1. *In vitro* efficacy of different PGPRs against *X. axonopodis* pv *glycines*

PGPR strain	Inhibition (mm)[a]		
	10^4 c.f.u ml-[1]	10^6 c.f.u ml-[1]	10^8 c.f.u ml-[1]
PGPR-1	5.13[a]	9.42[cd]	14.24[eh]
PGPR-2	2.18[b]	5.92[a]	8.46[cd]
PGPR-3	11.91[d]	23.62[f]	32.45[k]
PGPR-4	8.31[cd]	21.46[g]	31.52[k]
PGPR-5	7.63[c]	15.58[h]	23.81[f]
PGPR-6	7.16[c]	13.82[h]	21.52[fg]
PGPR-7	7.88[c]	16.31[h]	24.27[j]
PGPR-8	9.34 [cd]	19.25[i]	28.14
PGPR-9	3.52[b]	5.65[a]	8.92[cd]
PGPR-10	12.35[de]	23.15[fj]	33.51
PGPR-11	7.21[c]	16.18[hi]	22.53[f]
PGPR-12	9.75[cd]	21.32[fg]	31.15[k]
Control	0	0	0
SEm±	0.232	0.499	0.767
LSD (P = 0.05)	0.476	1.026	1.578

[a] Mean of three replications. Values in a column superscribed by same letter(s) are not significantly different *(P* = 0.05) by LSD test.

Table 2. Effect of different PGPRs on germination of *X. axonopodis* pv *glycines* inoculated seeds and growth parameters of seedling

Treatment	Seed germination[W] (%)[X]	Average shoot length[W]	Average (cm) root length[W]	Average (cm)fresh weight[W] (g)	Average dry weight[W] (mg)	Vigor index[W]
PGPR-1	99.2 (84.87)[a]	7.25[d]	6.95[de]	3.48[e]	520[c]	1408.64
PGPR-2	94.3 (76.18)[bc]	7.88[c]	6.79[c]	4.25[b]	470[a]	1383.38
PGPR-3	94.5 (76.31)[bc]	5.85[bc]	9.66[b]	5.45[a]	730[a]	1465.69
PGPR-4	100 (90.00)[a]	7.45	7.10	4.25[b]	610[b]	1455.00
PGPR-5	95.6 (77.89)[b]	8.10[a]	9.25[b]	5.25[e]	495[b]	1658.66
PGPR-6	92.1 (73.68)[c]	4.35	6.26[e]	3.25[ef]	345[d]	977.18
PGPR-7	98.8 (83.71)[b]	5.54[a]	8.16[b]	3.45[c]	385[d]	1353.56
PGPR-8	93.5 (75.23)[bc]	6.54$_d$	6.01[c]	4.15[bc]	470[c]	1173.43
PGPR-9	91.2 (72.74)[c]	3.34-[e]	5.96[e]	3.54[ef]	384[d]	848.16
PGPR-10	92.5 (74.11)[bc]	5.75[e]	5.50[d]	3.50[f]	340	1040.63
PGPR-11	98.4 (82.73)[bc]	7.21[ab]	7.78[ab]	4.12[cd]	608[b]	1475.02
PGPR-12	100 (90.00)[a]	9.36[a]	8.12[a]	5.07[d]	690[a]	1748.00
Uninoculated control	84.3 (66.66)	7.40	8.05[b]	4.47[a]	520[c]	1302.44
Inoculated control	70.4 (57.04)	6.25[e]	7.25[d]	4.41	490	950.40
SEm±	2.050	0.160	0.192	0.088	7.801	
LSD *(P=0.05)*	4.199	0.328	0.394	0.180	15.977	

[W] Mean percentage of seed germinated after 10 days of treatment challenge inoculated with *X. axonopodis* pv. *glycines* of three replications having 10 seeds per each replication; [X] For percentages, the analysis was based on the angular transformation. Values in a column superscribed by same letter(s) are not significantly different (P = 0.05) by LSD test; [Y] PVI = (mean epicotyl length+ mean hypocotyl length) x % germination

Table 3. Efficacy of bioagents (PGPRs) against *X. axonopodis* pv. *glycines* under field condition

Treatment	PDI[W] (%)[X]	PDC[W](%)[X]	Yield (q/ha) [W]	Per cent increase in yield[W]
Tetracycline	9.13 (17.58)	77.79	11.32	38.89
PGPR-1	27.75 (31.79)	32.51	8.80	07.97
PGPR-4	20.92 (27.21)	49.29	9.71[ab]	19.14
PGPR-5	17.42 (24.66)[a]	57.63	10.15[b]	24.54
PGPR-7	22.73 (28.47)	44.72	9.38 [a]	15.09
PGPR-12	15.81 (23.42)[a]	61.55	10.02[b]	30.31
Un treated control	41.12 (39.88)	00.00	8.15	00.00
SEm±	0.856		0.253	
LSD (P = 0.05)	1.759		0.521	

[W] Pooled mean percentage of two-year field trial (2000 & 2001) challenge inoculated with *X. axonopodis* pv. *glycines*. Each trial has three replications of each treatment with 50 plants per each replication; [X] For percentages, the analysis was based on the angular transformation. Values in a column superscribed by same letter(s) are not significantly different (P= 0.05) by LSD test

leaves against *X. oryzae* pv. *oryzae* (Tong *et al.*, 1999), *B. polymyxa* BP1 isolated from cauliflower seeds against *X. campestris* pv. *campestris* (Assis *et al.*, 1995; Pichard and Thouvenot, 1999) similar to kasugamycin and *B. subtilis* isolates *in vitro* as well as *in vivo*. Similar, PGPRs isolates have been reported to be effective in cluster bean against *X. axonopodis* pv. *cymopsidis,* cauliflower against *X. campestris* pv. *campestris* (Sain and Gour, 2005, 2009, Sain *et al.*, 2007).

Efficacy of PGPRs under field conditions

Field experiments revealed that the PGPR-12 showed significantly (LSD < P 0.05) maximum PDC and highest yield (Q/ha) over other treatments (Table 3). Although the highest yield was observed with the tetracycline treatment, whereas, PGPR–5, PGPR–4 and PGPR–7 also showed considerably higher decrease in percent disease index and increase in yield as compared to control. Furthermore, these treatments are statistically (LSD < P 0.05) at par as regards increase in yield resulting at both the 5 and 1% degree of freedom. Good control of bacterials pustules of soybean by applying *Pseudomonas* sp. (rhizoplane) as seed treatment and two sprays (Dzhalilov *et al.*, 1994) and bacterial blight of cluster bean by seed treatment with *B. subtilis* and spray of antibiotics streptomycin at 35 and 49 DAS the field has been reported (Lodha, 2001). Similar, PGPRs treatments has been reported to be effective under field conditions on cluster bean against *X. axonopodis* pv. *cymopsidis* and on cauliflower against *X. campestris* pv. *campestris* (Sain and Gour, 2005, 2009; Sain *et al.*, 2007).

The results obtained with the treatments of PGPRs with regard to seed germination, dry weight and PVI were different to the trend as observed in *in vitro* bacterial growth inhibition experiments. In conclusion, the present investigation showed that for selecting an effective PGPRs or biocontrol agent in successful disease management into the field conditions, not only bacterial growth inhibition tests but also the seedling vigor index and including field trials are very much important. Thus the best performing PGPRs could be used in successful disease management or can be used in integrated disease management strategy with most successful treatment combination of PGPR 12, PGPR–7 and PGPR–5 which showed considerably decrease in % disease index and increase in yield against bacterial pustules caused by *X. campestris* pv. *glyciness.*

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